

**PCT**WORLD INTELLECTUAL PROPERTY ORGANIZATION  
International Bureau

## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>6</sup> :</b> A01N 43/04, C12Q 1/68, C12P 21/06, C12N 15/00	<b>A1</b>	<b>(11) International Publication Number:</b> <b>WO 98/38861</b> <b>(43) International Publication Date:</b> 11 September 1998 (11.09.98)
<b>(21) International Application Number:</b> PCT/US98/04128 <b>(22) International Filing Date:</b> 3 March 1998 (03.03.98)  <b>(30) Priority Data:</b> 08/810,540 3 March 1997 (03.03.97) US  <b>(63) Related by Continuation (CON) or Continuation-in-Part (CIP) to Earlier Application</b> US 08/810,540 (CIP) Filed on 3 March 1997 (03.03.97)  <b>(71) Applicant (for all designated States except US):</b> THE TRUSTEES OF COLUMBIA UNIVERSITY IN THE CITY OF NEW YORK [US/US]; West 116th Street and Broadway, New York, NY 10027 (US).  <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> TROY, Carol, M. [US/US]; 40 Euclid Avenue, Hastings-on-Hudson, NY 10706 (US). SHELANSKI, Michael, L. [US/US]; 241 Kane Street, Brooklyn, NY 11231 (US).  <b>(74) Agent:</b> WHITE, John P.; Cooper & Dunham LLP, 1185 Avenue of the Americas, New York, NY 10036 (US).		<b>(81) Designated States:</b> AU, CA, JP, MX, US, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).  <b>Published</b> <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
<b>(54) Title:</b> ANTISENSE COMPOUNDS WHICH PREVENT CELL DEATH AND USES THEREOF		
<b>(57) Abstract</b>  The present invention provides for an antisense oligonucleotide having the sequence 5'GCTCGGCGCCGCCATTTCAG3'. The invention also provides for an antisense oligonucleotide having the sequence 5'GTCAGCGGCCATCAGCTT3'. The present invention further provides for a method for treating a neurodegenerative disorder in a subject which comprises administering to the subject a compound in an amount effective to inhibit neuronal cell death and thus treat the neurodegenerative disorder in the subject, which compound comprises the oligonucleotide 5'GCTCGGCGCCGCCATTTCAG3' and a delivery agent. The present invention provides for a method of inhibiting trophic factor withdrawal mediated death of a cell which comprises contacting the cell with an amount of the oligonucleotide 5'GCTCGGCGCCGCCATTTCAG3' effective to inhibit death of the cell.		

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece			TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon		Republic of Korea	PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakhstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

ANTISENSE COMPOUNDS WHICH PREVENT CELL DEATH AND USES THEREOF

5 This application is a continuation-in-part of U.S. Serial No. 08/810,540, filed March 3, 1997, the contents of which are hereby incorporated by reference into the present application.

10 The invention disclosed herein was made with Government support under NIH Grants No. and NS15076 from the Department of Health and Human Services. Accordingly, the U.S. Government has certain rights in this invention.

Background of the Invention

15 Throughout this application, various publications are referenced by author and date. Full citations for these publications may be found listed alphabetically at the end of the specification immediately preceding the claims. The  
20 disclosures of these publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art as known to those skilled therein as of the date of the invention described and claimed herein.

25 Neuronal death by apoptosis is a normal feature of development in which it appears that the death program is triggered by the failure of a given neuron to compete for limiting supplies of target-derived neurotrophic factors. Neurons also undergo  
30 apoptotic death in the post-developmental period when deprived of appropriate trophic factors or when subjected to any of a variety of stresses and injuries. Apoptosis also accounts for at least a portion of cellular loss in degenerative neurological diseases including Alzheimer's disease and  
35 amyotrophic lateral sclerosis (Coyle and Puttfarcken, 1993; Schapira, 1995; Williams, 1995; Brown, 1995).

-2-

Neuronal apoptotic death may be precipitated by widely different initiating causes. In the rat pheochromocytoma PC12 line, a commonly used model for neuronal differentiation and cell death, apoptosis may be triggered by either trophic factor/NGF withdrawal as well as by sympathetic neurons (Greene, 1978; Rukenstein et al., 1991; Mesner et al., 1992; Pittman et al., 1993; Lindenboim et al., 1995; Batistatou and Greene, 1991) or oxidative stress induced by down-regulation of Cu<sup>++</sup>/Zn<sup>++</sup> superoxide dismutase (SOD1) (Troy and Shelanski, 1994; Troy et al., 1996a,b,c). The initiating mechanisms of death appear to be distinct in each instance. Apoptosis triggered by nerve growth factor (NGF) deprivation is blocked by cAMP analogs (Rydel and Greene, 1988; Rukenstein et al., 1991) and high concentrations of N-acetyl cysteine (Ferrari et al., 1995) whereas these agents do not inhibit death induced by down regulation of superoxide dismutase (SOD1) (Troy et al., 1996 a,c). In contrast, the latter is blocked by vitamin E (Troy and Shelanski, 1994) and inhibitors of nitric oxide (NO) synthase (Troy et al. 1996a), which have no effect on apoptosis evoked by NGF withdrawal (Ferrari et al., 1995; Farinelli et al., 1996). Despite these initial mechanistic differences, there is evidence for common or similar downstream elements in the pathways that lead to death in both paradigms.

25

Summary of the Invention

The present invention provides for an antisense  
5 oligonucleotide having the sequence 5'GCTCGGCGCCGCCATTTCCAG3'.  
The invention also provides for an antisense oligonucleotide  
having the sequence 5'GTCAGCGGCCATCAGCTT3'. The present  
invention further provides for a method for treating a  
neurodegenerative disorder in a subject which comprises  
10 administering to the subject a compound in an amount effective  
to inhibit neuronal cell death and thus treat the  
neurodegenerative disorder in the subject, which compound  
comprises the oligonucleotide 5'GCTCGGCGCCGCCATTTCCAG3' and a  
delivery agent. The present invention provides for a method  
15 of inhibiting trophic factor withdrawal mediated death of a  
cell which comprises contacting the cell with an amount of the  
oligonucleotide 5'GCTCGGCGCCGCCATTTCCAG3' effective to inhibit  
death of the cell.

-4-

Brief Description of the Figures

Figures 1A, 1B, 1C and 1D. Down-regulation of Nedd2 in PC12 cells by Penetratin1-linked antisense oligonucleotide to Nedd2 (V-ANedd).

Naive and neuronally differentiated PC12 cells (pretreated for at least 7 days with NGF, nerve growth factor) were plated on Matrigel coated multichamber slides. V-ANedd (400 nM) was added to the indicated cultures after plating. Naive cultures were grown in RPMI 1640 medium with 5% FCS and 10% horse serum; neuronal cultures were grown in RPMI 1640 medium with 1% horse serum and NGF (100 ng/ml). After overnight maintenance, cells were fixed in ice cold methanol and then immunostained with anti-N-Nedd. Cells were observed with a Nikon® fluorescence microscope. x120.

Figures 2A, 2B, 2C and 2D. Cellular localization of Nedd2 before and after trophic factor deprivation.

Naive and neuronally differentiated were grown with serum and serum and NGF respectively. The cells were then plated in serum-free RPMI 1640 medium for 20 hrs. with or without NGF as indicated. Cultures were stained with anti-N-Nedd and were observed with a BioRad® MRC600 confocal microscope. x1600.

Figures 3A, 3B. Regulation of Nedd2 by V-ANedd.

Naive PC12 cells were grown for 24 hours in serum-free RPMI 1640 medium in the presence or absence of NGF (100 ng/ml) and with or without V-ANedd (400 nM) or V-SNedd (400 nM) as indicated. The cells were extracted in sample buffer, the extracts boiled and equal amounts of protein resolved by 10% SDS-PAGE and transferred to nitrocellulose. Blots were probed with (Fig 3A) anti-N-Nedd at 1:500 or (Fig 3B) anti-C-Nedd at

-5-

1:330 and staining visualized with ECL. Bands were quantified using Scion Image® software and normalized against peripherin levels. The level of down-regulation is representative of that obtained in 5 independent experiments.

5

Figures 4A, 4B and 4C. V-ANedd rescues PC12 cells from serum deprivation but not from SOD1 down-regulation.

(Fig 4A) V-ANedd differentially protects PC12 cells from serum deprivation. For serum deprivation (4 left-hand bars) cells were extensively washed as described in Experimental Procedures hereinbelow and the indicated additives (100 ng/ml NGF, 400 nM V-ANedd, or 200 nM V-ICE<sub>inh</sub>) were added at the time of plating in serum-free RPMI1640 medium. For SOD1 down-regulation (4 right-hand bars), PC12 cells were re-plated on fresh collagen-coated 24-well dishes in complete medium (RPMI 1640 medium with 10% horse serum, 5% fetal bovine serum) with 50 nM V-ASOD1 (vector linked antisense oligonucleotide to SOD1). Additives (800 nM V-ANedd, 25 nM V-ICE<sub>inh</sub>) were included as indicated. Control cells were in complete medium. Cultures were incubated for 24 hours, lysed and number of intact nuclei counted. The numbers of surviving cells are expressed relative to the number in the control cultures (designated as 100). Here, as in past studies (Greene and Tischler, 1976; Rukenstein et al., 1991; Troy et al., 1996a,b), NGF or complete medium promotes survival of all cells initially plated. Experiments were performed in triplicate wells and data are expressed as means ± SEM.

(Fig 4B) Dose-response curve for protection from serum deprivation by V-ANedd. PC12 cells were washed for trophic factor deprivation and plated in serum-free medium with the indicated concentrations of V-ANedd. Cell survival relative to the number present with the addition of NGF was measured at one day.

(Fig 4C) V-ANedd does not block the V-ASOD1-induced increase

-6-

of IL-1 $\beta$  production. PC12 cells were plated with the indicated additives (50 nM V-ASOD1, 25 nM V-ICE<sub>inh</sub>, 800 nM V-ANedd). Controls contained complete medium. After 20 hours, media were removed and IL-1 $\beta$  was measured by ELISA using the Intertest-1 $\beta$ X<sup>®</sup> kit. Data are expressed as means  $\pm$  SEM (n=3).

Figures 5A, 5B and 5C. Neuronally differentiated PC12 cells are rescued from NGF deprivation, but not from SOD1 down-regulation by V-ANedd.

(Fig 5A) V-ANedd differentially protects neuronally differentiated PC12 cells from apoptosis caused by NGF withdrawal. PC12 cells were neuronally differentiated by exposure to NGF (100 ng/ml) for at least 7 days in RPMI 1640 medium plus 1% horse serum. Cells were deprived of serum and NGF and re-plated as described in Figures 4A-C. Additives present at the time of plating included 400 nM V-ANedd, 400 nM V-ICE<sub>inh</sub> or 100 ng/ml NGF (4 left-hand bars). For SOD1 down-regulation (4 right-hand bars) neuronally differentiated PC12 cells were plated in RPMI 1640 medium plus 1% horse serum, with 100 ng/ml NGF. At the time of plating, cultures were incubated, as indicated, with 50 nM V-ASOD1 and with the indicated additives (800 nM V-ANedd, 50 nM V-ICE<sub>inh</sub>). Cell survival was determined after one day and expressed as in Figures 4A-C.

(Fig 5B) Dose-response curve for protection from NGF deprivation by V-ANedd. Neuronally differentiated PC12 cells were washed as above for NGF deprivation and plated in serum-free medium with the indicated concentrations of V-ANedd. Cell survival relative to the number present with the addition of NGF was quantified at one day.

(Fig 5C) V-ANedd provides long term protection against NGF deprivation. Neuronally differentiated PC12 cells were deprived of NGF and serum and plated as described in Figure



-7-

5A. V-ANedd (400 nM) was included at the time of NGF deprivation and replenished 1 day later. Cell survival was determined at the indicated times as in Figures 4A-C.

5 Figures 6A-6B. V-ANedd protects sympathetic neurons from NGF withdrawal, but not from oxidative stress.

(Fig 6A) V-ANedd protects sympathetic neurons from NGF-withdrawal. At the time of NGF deprivation, V-ANedd (400  
10 nM) was added to the cultures as indicated. Numbers of surviving neurons were determined at the indicated times as described in Experimental Procedures hereinbelow and are reported as relative to the number present in each culture at the time of NGF withdrawal.

15 (Fig 6B) V-ANedd does not protect sympathetic neurons from death induced by SOD1 down-regulation and nitric oxide generation. Sympathetic neurons, after 6 days in culture, were maintained with NGF (100 ng/ml) and mixtures of the following additives as indicated: V-ASOD1 (50 nM), SNAP (100  
20  $\mu$ M) and V-ANedd (400 nM). Numbers of surviving neurons were determined at the indicated times as above.

Figures 7A-7I. Morphology of cells rescued by V-ANedd.

25 Photomicrographs of cells treated as described in the preceding figures. Figs 7A-C, naive PC12 cells: Fig 7A, in serum-free RPMI 1640 medium with 100 ng/ml NGF (24 hr); Fig 7B, in RPMI 1640 medium alone (24 hr); Fig 7C, in serum-free RPMI 1640 medium with 400 nM V-ANedd (24 hr). Figs 7D-F,  
30 neuronally differentiated PC12 cells Fig 7D, re-plated in serum-free RPMI 1640 medium with 100 ng/ml NGF (24 hr); Fig 7E, re-plated in serum-free RPMI 1640 medium without NGF (24 hr); Fig 7F, re-plated in serum-free RPMI 1640 medium with 400 nM V-ANedd (24 hr). Figs 7G-I, sympathetic neurons: Fig 7G,  
35 cultured with 100 ng/ml NGF (3 days); Fig 7H, cultured in

-8-

NGF-free medium with anti-NGF (3 days); Fig 7I, cultured in NGF-free medium with anti-NGF plus 400 nM V-ANedd (3 days). Phase contrast optics. x180.

- 5 Figure 8. The human form of Nedd2, Ich1 or caspase 2, was down-regulated in Jurkat cells, a human lymphoma cell line, and found to protect from anti-Fas mediated death in a dose-dependent manner. V-ICH is an antisense oligonucleotide having an antisense sequence of human Ich, specifically V-AICH
- 10 has the sequence of Seq ID No. 2.

Detailed Description of the Invention

The present invention provides for an oligonucleotide having the sequence 5'GCTCGGCGCCGCCATTTCCAG3' (SEQ ID No. 1). The present invention also provides for an oligonucleotide having the sequence 5'GTCAGCGGCCATCAGCTT3' (SEQ. ID No. 2). The present invention also provides for variants of these oligonucleotide sequences which conserve their biological activity. It is known to one of skill in the art that certain variations of nucleotides in an oligonucleotide will not affect the biological activity. The present invention provides for an oligonucleotide which is capable of inhibiting the expression of a Nedd2 rodent gene or inhibiting the expression of a human Ich-1L gene by antisense inhibition of translation. The full sequence of Ich-1L has been deposited in GenBank at accession number (code) U13021. The oligonucleotide may comprise DNA, cDNA or synthetic oligonucleotides.

The present invention also provides for a compound comprising the oligonucleotide having the sequence 5'GCTCGGCGCCGCCATTTCCAG3' (SEQ ID No. 1) and an agent capable of specifically directing the compound to a cell. The present invention also provides for a compound comprising the oligonucleotide having the sequence 5'GTCAGCGGCCATCAGCTT3' (SEQ. ID No. 2) and an agent capable of specifically directing the compound to a cell. The compound may comprise any variants of the sequence 5'GCTCGGCGCCGCCATTTCCAG3' (SEQ ID No. 1) or of the sequence 5'GTCAGCGGCCATCAGCTT3' (SEQ. ID No. 2) which do not change their respective biological activity. The agent may comprise a polypeptide comprising at least a portion of an Antennapedia polypeptide. In one embodiment of the present invention, the agent comprises at least a portion of a polypeptide comprising the sequence NPyS-Arg-Gln-Ile-Lys-Ile-Trp-Phe-Gln-Asn-Arg-Arg-Met-Lys-Trp-Lys-Lys- (Seq. I.D. No.

-10-

3).

As used herein, biological activity of 5'GCTCGGCGCCGCCATTTCCAG3' (SEQ ID No. 1) comprises any antisense activity particular to this oligonucleotide, inhibition of the rodent Nedd2 gene, binding to and inhibiting translation of Nedd2 mRNA, binding to and inhibiting transcription of Nedd2 DNA or cDNA, inhibiting the expression of any protein of the interleukin-1 converting enzyme family member, inhibiting cell death, inhibiting apoptosis.

As used herein, biological activity of 5'GTCAGCGGCCATCAGCTT3' (SEQ. ID No. 2) comprises any antisense activity particular to this oligonucleotide, inhibition of the human Ich-1L gene, binding to and inhibiting translation of Ich-1L mRNA, binding to and inhibiting transcription of Ich-1L DNA or cDNA, inhibiting the expression of any protein of the interleukin-1 converting enzyme family member, inhibiting cell death, inhibiting apoptosis.

In another embodiment of the present invention, the compound may comprise NPyS-Arg-Gln-Ile-Lys-Ile-Trp-Phe-Gln-Asn-Arg-Arg-Met-Lys-Trp-Lys-GCTCGGCGCCGCCATTTCCAG (SEQ ID No. 4).

The agent may comprise an antibody, an adjuvant or a cell-specific ligand. The agent may further comprise Penetratin<sup>®</sup>.

In the practice of the present invention, the cell may be a neuronal cell.

The present invention also provides for a pharmaceutical composition comprising an oligonucleotide having the sequence 5'GCTCGGCGCCGCCATTTCCAG3' (SEQ ID No. 1) or variants thereof and a pharmaceutically acceptable carrier. The present invention provides for a pharmaceutical composition comprising

-11-

an oligonucleotide having the sequence 5'GTCAGCGGCCATCAGCTT3' (SEQ. ID No. 2) or variants thereof and a pharmaceutically acceptable carrier.

- 5 The carrier may comprise a diluent, an appropriate adjuvant, a herpes virus, a liposome, a microencapsule, a neuronal cell receptor ligand, a neuronal-specific virus, a polymer encapsulated cell or a retroviral vector.
- 10 The pharmaceutically acceptable carrier may be in the form of an aerosol, intravenous, oral or topical carrier.

The present invention also provides for a method of inhibiting trophic factor withdrawal mediated death of a cell which  
15 comprises contacting the cell with an amount of the oligonucleotide having the sequence 5'GCTCGGCGCCGCCATTTCCAG3' (SEQ ID No. 1) effective to inhibit death of the cell.

The cell may be found in a subject. The subject may be a  
20 human. The cell may be a brain cell, a spinal cord cell, neuronal cell.

The agent may comprise a polypeptide encompassing at least a portion of an Antennepedia polypeptide. The agent may include  
25 a polypeptide having the sequence NPyS-Arg-Gln-Ile-Lys-Ile-Trp-Phe-Gln-Asn-Arg-Arg-Met-Lys-Trp-Lys-Lys- (Seq. I.D. No. 3). The agent may comprise an antibody, an adjuvant or a cell-specific ligand.

- 30 The present invention also provides for a method for treating a neurodegenerative disorder in a subject which comprises administering to the subject a compound which comprises an oligonucleotide having the sequence 5'GCTCGGCGCCGCCATTTCCAG3' (SEQ ID No. 1) or 5'GTCAGCGGCCATCAGCTT3' (SEQ. ID No. 2) or  
35 any variants thereof, and an agent capable of directing

-12-

delivery of the compound to a cell, the compound being present in an amount effective to inhibit neuronal cell death and thus treat the neurodegenerative disorder in the subject.

5 In the practice of this invention the neurodegenerative disorder may associated with aging, amyotrophic. lateral sclerosis, Alzheimer's disease, dentatorubral and pallidolysian atrophy, Huntington's disease, Machado-Joseph disease, multiple sclerosis, muscular dystrophy, Parkinson's  
10 disease, senility, spinocerebellar ataxia type I, spinobulbar muscular atrophy, stroke, trauma.

The administration may comprise aerosol delivery; intralesional, intraperitoneal, intramuscular or intravenous  
15 injection; infusion; liposome-mediated delivery; anal, nasal, oral, ocular, otic or topical delivery of the pharmaceutical composition.

One of skill in the art would know methods of introducing a  
20 compound or oligonucleotide of the present invention into a cell or into a subject. Methods of incorporation of nucleic acid molecules into liposomes are well known to those of ordinary skill in the art. In another embodiment of this method, the oligonucleotide may be delivered via transfection,  
25 injection, or viral infection. There are several protocols for human gene therapy which have been approved for use by the Recombinant DNA Advisory Committee (RAC) which conform to a general protocol of target cell infection and administration of transfected cells (see for example, Blaese, R.M., et al.,  
30 1990; Anderson, W. F., 1992; Culver, K.W. et al., 1991). In addition, U.S. Patent No. 5,399,346 (Anderson, W. F. et al., issued March 21, 1995) describes procedures for retroviral gene transfer. The contents of these support references are incorporated in their entirety into the subject application.  
35 Retroviral-mediated gene transfer requires target cells which

-13-

are undergoing cell division in order to achieve stable integration hence, cells are collected from a subject often by removing blood or bone marrow.

5 Several methods have been developed over the last decade for the transduction of genes into mammalian cells for potential use in gene therapy. In addition to direct use of plasmid DNA to transfer genes, episomal vectors, retroviruses, adenoviruses, parvoviruses, and herpesviruses have been used  
10 (Anderson et al., 1995; Mulligan, 1993; The contents of which are incorporated in their entirety into the subject application). For transfer of genes into cells ex vivo and subsequent reintroduction into a host, as would be most feasible in immunodeficiency patients, retroviruses have been  
15 the vectors of choice.

Throughout this application, the following standard abbreviations are used throughout the specification to indicate specific nucleotides:

20

C=cytosine

A=adenosine

T=thymidine

G=guanosine

The DNA molecules and the oligonucleotides of the subject  
25 invention also include DNA molecules coding for polypeptide analogs, fragments or derivatives of oligonucleotides which differ from naturally-occurring forms in terms of the identity or location of one or more nucleotide bases (deletion analogs containing less than all of the bases specified for the  
30 molecule, substitution analogs wherein one or more bases specified are replaced by other bases and addition analogs wherein one or more nucleotide bases is added to a terminal or medial portion of the oligonucleotide) and which share some or all properties of naturally-occurring forms. These include:  
35 the incorporation of bases "preferred" for mammalian hosts;

-14-

the provision of sites for cleavage by restriction endonuclease enzymes; and the provision of additional initial, terminal or intermediate DNA sequences that facilitate construction of readily expressed vectors.

5

The compound of the present invention may have increased stability, efficacy, potency and bioavailability by virtue of the agent or by virtue of the oligonucleotide. Further, the compound may have decreased toxicity. The compound may have enhanced mucosal intestinal permeability. The compound may be synthetically prepared. The compound of the present invention may include L-, D- or unnatural amino acids, alpha, alpha-disubstituted amino acids, N-alkyl amino acids, lactic acid (an isoelectronic analog of alanine). The peptide backbone of the compound may have at least one bond replaced with PSI-[CH=CH] (Kempf et al. 1991). The compound may further include trifluorotyrosine, p-Cl-phenylalanine, p-Br-phenylalanine, poly-L-propargylglycine, poly-D,L-allyl glycine, or poly-L-allyl glycine.

20

One embodiment of the present invention is a peptidomimetic compound having the biological activity of the structure NPyS-Arg-Gln-Ile-Lys-Ile-Trp-Phe-Gln-Asn-Arg-Arg-Met-Lys-Trp-Lys-GCTCGGCGCCGCCATTCCAG (SEQ ID No. 4) or NPyS-Arg-Gln-Ile-Lys-Ile-Trp-Phe-Gln-Asn-Arg-Arg-Met-Lys-Trp-Lys-Lys-GTCAGCGGCCATCAGCTT (SEQ. ID No. 5) wherein the compound has a bond, a peptide backbone or an amino acid component replaced with a suitable mimic. Examples of unnatural amino acids which may be suitable amino acid mimics include  $\beta$ -alanine, L- $\alpha$ -amino butyric acid, L- $\gamma$ -amino butyric acid, L- $\alpha$ -amino isobutyric acid, L- $\epsilon$ -amino caproic acid, 7-amino heptanoic acid, L-aspartic acid, L-glutamic acid, cysteine (acetamindomethyl), N- $\epsilon$ -Boc-N- $\alpha$ -CBZ-L-lysine, N- $\epsilon$ -Boc-N- $\alpha$ -Fmoc-L-lysine, L-methionine sulfone, L-norleucine, L-norvaline, N- $\alpha$ -Boc-N- $\delta$ -CBZ-L-ornithine, N- $\delta$ -Boc-N- $\alpha$ -CBZ-L-

35



-15-

ornithine, Boc-p-nitro-L-phenylalanine, Boc-hydroxyproline, Boc-L-thioprolin. (Blondelle, et al. 1994; Pinilla, et al. 1995).

5 Another embodiment of the present invention is a method for alleviating symptoms of a neurodegenerative disorder in a subject which includes administering to the subject the compounds described hereinabove, the compound being present in an amount effective to inhibit neuronal cell death and thus  
10 alleviate the symptoms of the neurodegenerative disorder in the subject.

The neurodegenerative disorder may be associated with aging, Alzheimer's disease, amyotrophic lateral sclerosis, multiple  
15 sclerosis, muscular dystrophy, Parkinson's disease, senility, spinocerebellar ataxia type I, spinobulbar muscular atrophy, stroke, trauma. The subject may be a mammal. The mammal may be a human. The administration may include aerosol delivery; intralesional, intraperitoneal, intramuscular or intravenous  
20 injection; infusion; liposome-mediated delivery; anal, nasal, oral, ocular, otic or topical delivery of the pharmaceutical composition.

The effectiveness of the compounds described herein to prevent  
25 trophic factor withdrawal mediated cell death is a surprising result that would not have been anticipated by one skilled in the art. The specific sequences used in the antisense oligonucleotides as described herein provide unexpected results in the prevention and inhibition of cell death  
30 mediated by trophic factor withdrawal.

As used herein "trophic factor" encompasses the family of neurotrophins. Some examples of neurotrophic factors are NGF, BDNF, NT3, NT4, GDNF. Other neurotrophic factors and trophic  
35 factors would be known to one of ordinary skill in the art.

-16-

Also provided by the invention are pharmaceutical compositions comprising therapeutically effective amounts of polypeptide products of the invention together with suitable diluents, preservatives, solubilizers, emulsifiers, adjuvants and/or carriers. A "therapeutically effective amount" as used herein refers to that amount which provides a therapeutic effect for a given condition and administration regimen. Such compositions are liquids or lyophilized or otherwise dried formulations and include diluents of various buffer content (e.g., Tris-HCl., acetate, phosphate), pH and ionic strength, additives such as albumin or gelatin to prevent absorption to surfaces, detergents (e.g., Tween 20, Tween 80, Pluronic F68, bile acid salts), solubilizing agents (e.g., glycerol, polyethylene glycerol), anti-oxidants (e.g., ascorbic acid, sodium metabisulfite), preservatives (e.g., Thimerosal, benzyl alcohol, parabens), bulking substances or tonicity modifiers (e.g., lactose, mannitol), covalent attachment of polymers such as polyethylene glycol to the protein, complexation with metal ions, or incorporation of the material into or onto particulate preparations of polymeric compounds such as polylactic acid, polyglycolic acid, hydrogels, etc, or onto liposomes, microemulsions, micelles, unilamellar or multilamellar vesicles, erythrocyte ghosts, or spheroplasts. Such compositions will influence the physical state, solubility, stability, rate of *in vivo* release, and rate of *in vivo* clearance. The choice of compositions will depend on the physical and chemical properties of the protein having the activity of inhibiting neuronal cell death. For example, a product derived from a membrane-bound form of the protein may require a formulation containing detergent. Controlled or sustained release compositions include formulation in lipophilic depots (e.g., fatty acids, waxes, oils). Also comprehended by the invention are particulate compositions coated with polymers (e.g., poloxamers or poloxamines) and the compound coupled to antibodies directed against tissue-

-17-

specific receptors, ligands or antigens or coupled to ligands of tissue-specific receptors. Other embodiments of the compositions of the invention incorporate particulate forms protective coatings, protease inhibitors or permeation enhancers for various routes of administration, including  
5 parenteral, pulmonary, nasal, oral, injection or infusion by intravenous, intraperitoneal, intracerebral, intramuscular, intraocular, intraarterial or intralesional.

10 The present invention includes a delivery system which links the polypeptide to an agent which directs the polypeptide to neuronal cells in order to facilitate entry into the cells. The Antennepedia protein may be used as such a delivery agent. The invention also includes therapeutic uses of the isolated  
15 polypeptide to prevent trophic factor withdrawal mediated cell death. Related therapeutic uses include treating stroke, trauma, neurodegenerative disorders or regenerating neurons, cardiac ischemia, liver disease, pulmonary disease, congestive heart disease, myocardial infarction, ALS, Alzheimer's  
20 disease, Parkinson's disease, senility, aging, muscular dystrophy, multiple sclerosis, Huntington's disease, spinocerebellar ataxia type I, Machado-Joseph disease, spinobulbar muscular atrophy or dentatorubral and pallidolysian atrophy.

25 This invention is illustrated in the Experimental Detail section which follows. These sections are set forth to aid in an understanding of the invention but are not intended to, and should not be construed to, limit in any way the invention as  
30 set forth in the claims which follow thereafter.

-18-

EXPERIMENTAL DETAILSEXAMPLE 1:Nedd2 IS REQUIRED FOR APOPTOSIS FOLLOWING TROPHIC  
FACTOR WITHDRAWAL BUT NOT SOD1 DOWN-REGULATION IN SYMPATHETIC  
5 NEURONS AND PC12 CELLS

Abstract: Differential requirements for Nedd2 in apoptosis

10 Activation of cysteine aspartases (caspases) appears to be a  
required element of apoptotic death in many paradigms. It has  
been shown that general inhibitors of cysteine aspartases  
block apoptosis of PC12 cells and sympathetic neurons evoked  
by either trophic factor (NGF and/or serum) deprivation or  
15 SOD1 down-regulation. Moreover, activation of a caspase  
family member similar or equivalent to the interleukin-1 $\beta$   
converting enzyme (ICE) was implicated for death due to SOD1  
down-regulation, but not withdrawal of trophic support. The  
experiments presented here demonstrate that diminished  
20 expression of the cysteine aspartase Nedd2 in PC12 cells and  
sympathetic neurons induced by an appropriate  
vector-peptide-linked antisense oligonucleotide rescues them  
from death due to trophic factor deprivation without  
inhibiting apoptosis in the same cell types evoked by SOD1  
down-regulation. Neither the level (as revealed by western  
25 immunoblotting) nor the cellular distribution (as revealed  
immunohistochemically) of Nedd2 were demonstrably altered by  
trophic factor deprivation. However, evidence for proteolytic  
processing of Nedd2 (consistent with commencement of  
activation) was observed in PC12 cells after withdrawal of  
30 trophic support. These findings indicate that neuronal death  
triggered by different initial causes may be mediated by  
distinct members of the cysteine aspartase family.

In particular, inhibition studies implicate cysteine  
35 aspartases as obligate elements of the cell death mechanism in

-19-

both initiating causes of death (Troy et al., 1996b). However, even at the level of cysteine aspartases, findings have suggested the presence of parallel pathways. Apoptosis following SOD1 down-regulation is suppressed by the peptide YVAD, a potent inhibitor of the interleukin-1 converting enzyme (ICE), by blocking antibodies to IL-1 $\beta$  and by the IL-1 receptor antagonist IL-1Ra, while such agents have little or no effect on death caused by trophic factor/NGF withdrawal. These findings suggest that ICE itself, or another enzyme with pro-IL-1 $\beta$  cleaving activity is required for death in the SOD1 down-regulation paradigm whereas a different cysteine aspartase is required for death in the case of trophic factor deprivation (Troy et al., 1996b).

In light of the above, one object of the present study was to identify a specific cysteine aspartase that is required for neuronal apoptosis triggered by trophic factor deprivation. The cysteine aspartase Nedd2 is the rodent homologue of the human Ich-1/NEDD2 ICE-family member and is highly expressed in neurons and PC12 cells (Kumar et al., 1994). Over-expression of Nedd2/Ich-1 causes apoptosis in fibroblasts and neuroblastoma cells (Kumar et al., 1994) and expression of a NEDD2 antisense construct protects a hematopoietic-derived cell line from death evoked by cytokine deprivation (Kumar, 1995). In the experiments reported here, a designed vector-linked antisense oligonucleotide was used to suppress Nedd2 expression in cultured PC12 cells and sympathetic neurons. The findings herein indicate that Nedd2 plays a required role in neuronal apoptosis caused by loss of trophic support. In contrast, it does not appear to be required for death caused by SOD1 downregulation and thus, distinct cysteine aspartases mediate neuronal apoptosis triggered by different causes in the same cell.

-20-

**Materials and Methods - Cell Culture, PC12 cells**

PC12 cells were grown as previously described (Greene and Tischler, 1976) on rat-tail collagen-coated dishes in RPMI 1640 medium containing 5% fetal calf serum and 10% heat-inactivated horse serum (complete medium). NGF primed (neuronally differentiated) PC12 cells were grown for at least 7 days in RPMI 1640 medium plus 1% horse serum and NGF (100 ng/ml). For cell survival assays involving trophic factor deprivation, cells (either naive or NGF-pretreated) were extensively washed in serum-free RPMI 1640 medium and re-plated on fresh collagen-coated 24-well dishes as previously described (Rukenstein et al., 1991) in RPMI 1640 medium lacking serum or NGF. For SOD1 down-regulation survival assays, cells were re-plated in complete medium with V-ASOD1 (vector linked antisense oligonucleotide to SOD1, 50 nM), as previously described (Troy et al., 1996a). Various concentrations of V-ANedd (vector linked antisense oligonucleotide to Nedd2) were included in the medium as indicated. Numbers of viable cells per culture were determined by quantifying intact nuclei as previously described (Rukenstein et al., 1991). Counts were performed in triplicate and reported as means  $\pm$  SEM.

**Sympathetic neurons**

Sympathetic neuron cultures were prepared from 2 day old rat pups, as previously described (Ferrari et al., 1995). Cultures were grown in 24-well collagen coated dishes in RPMI 1640 medium plus 10% horse serum with mouse NGF (100 ng/ml). One day following plating, uridine and 5-fluorodeoxyuridine (10  $\mu$ M each) were added to the cultures and left for three days to eliminate non-neuronal cells. On the sixth day following plating NGF was removed by washing the cultures three times with RPMI 1640 medium plus 10% horse serum, followed by the addition of medium containing anti-mouse NGF (1:200, Sigma, St. Louis, MO) with or without V-ANedd. Each

-21-

culture was scored, as previously described (Rydel and Greene, 1988), as numbers of living, phase-bright neurons at various times. Three replicate cultures were assessed for each condition and data are normalized to numbers of neurons present in each culture at the time of NGF withdrawal and reported as mean  $\pm$  SEM.

#### Synthesis of V-ANedd

Oligonucleotides bearing an SH group at their 5' end and an NH group at their 3' end were purchased from Operon® (Alameda, CA). As previously described (Troy et al., 1996a) oligonucleotides were resuspended in deionized water, an equimolar ratio of Penetratin 1® (Oncor, Gaithersburg, MD) was added and the mixture was incubated at 37°C for 1 hour. The yield of the reaction, estimated by SDS-PAGE followed by Coomassie blue staining, was routinely above 50%. A scrambled sequence of the antisense oligonucleotide (same base composition, different order), defined as V-SNedd, was synthesized for use as a control.

20

#### Antibody preparation

Anti-N-Nedd2, a polyclonal rabbit antiserum was produced for us by Multiple Peptide Systems (San Diego, CA) using a 16 amino acid synthetic peptide homologous to the N-terminus (amino acids 1-16) as the antigen. The antiserum was affinity purified with peptide bound to Sulfo-Link® gel. Antiserum against a C-terminal peptide of Nedd2 (Nedd2 p12 C20) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

#### Immunofluorescence

PC12 cells were plated on coverslips or on 8 well multichamber slides (LabTek, VWR), coated with Matrigel®. After growth overnight, cells were fixed in ice cold methanol and then immunostained as described (Troy et al., 1990). The primary antibody was either affinity purified antibody Anti-N-Nedd2 or

35

-22-

Nedd2 p12 C20 (Santa Cruz Biotechnology) at a dilution of 1:200. The secondary antibody was fluorescein isothiocyanate-conjugated goat anti-rabbit (Cappel, Durham, NC) at 1:100. For visualization with a Nikon® fluorescence microscope slides were coverslipped with Aqua-mount®. Confocal microscopy was done on a BioRad® 600 confocal microscope.

#### Western blotting

PC12 cells grown with or without V-ANedd or V-SNedd were harvested in SDS-containing sample buffer and immediately boiled. Equal amounts of protein were separated by 10% PAGE, transferred to nitrocellulose and immunostained as described (Troy et al., 1992). The affinity purified anti-N-Nedd2 was used at a dilution of 1:500. The commercial antiserum, Nedd2 p12 C20 (Santa Cruz Biotechnology), was used at a dilution of 1:350. Visualization was with ECL using goat-anti-rabbit peroxidase at 1:1000. The relative intensity of the protein bands were quantified using Scion Image 1.55® software and samples were normalized by stripping and reprobing the blots with anti-peripherin antibody.

#### Assay of IL-1 $\beta$

IL-1 $\beta$  was quantified by ELISA using the Intertest-1 $\beta$ X® kit (Genzyme, Cambridge, MA) as previously described (Troy et al., 1996b). PC12 cells were grown as described above, on 24-well plates, in 500  $\mu$ l of medium. After one day incubation, medium was removed and IL-1 $\beta$  measured following the manufacturer's instructions, and number of viable cells in each well quantified.

#### Results

A vector-linked Nedd2 antisense oligonucleotide (V-ANedd) down-regulates Nedd2 protein



-23-

To suppress expression of Nedd2 in neuronal cells, an antisense oligonucleotide was designed corresponding to the last 12 bases in the 5' UTR and the first 9 bases in the coding region of the Nedd2 transcript (Kumar et al., 1994).  
5 The antisense oligonucleotide (ANedd; GCTCGGCGCCGCCATTTCCAG) (SEQ ID No. 1) is not homologous to any other reported mRNA sequence, including those of the other known cysteine aspartases. The oligonucleotide was linked to the vector peptide Penetratin 1<sup>®</sup> (V-) (Theodore et al., 1995; Troy et al., 1996a) to enhance its uptake by cells. The control  
10 scrambled oligonucleotide (SNedd; CCGTAGCGTAGCTCCGCCTGC) (SEQ ID No. 2) was also linked to vector peptide. This vector linked strategy significantly enhances the potency of antisense oligonucleotides and permits their use in the  
15 presence of serum (Troy et al., 1996a).

Using an affinity purified anti-peptide antiserum (anti-N-Nedd2) generated against a synthetic N-terminal Nedd2 peptide, the expression of Nedd2 was examined in naive and  
20 neuronal PC12 cells before and after exposure to V-ANedd. As revealed by immunohistochemistry, in control cells the anti-N-Nedd2 staining was primarily cytoplasmic. This decreased to almost undetectable levels when the cells were pretreated for 24 hours with 400 nM V-ANedd (Figures 1A-1D).  
25 In contrast, no change in staining was observed after exposure to 400 nM V-SNedd. Comparable results were found with another antibody generated to a C-terminal peptide of Nedd2. Confocal microscopy with either the N-terminal (Figures 2A-D) or the C-terminal antiserum demonstrated that the Nedd2 staining  
30 pattern does not change substantially after 20 hrs. of trophic factor deprivation (Figures 2A-D) in either naive or neuronal PC12 cells, or after SOD1 down-regulation. In all cases staining was largely cytoplasmic with 1-2 foci of staining seen in many nuclei. In the case of anti-N-Nedd2, all  
35 staining was abolished by preincubation with the immunizing

-24-

peptide.

By Western blot analysis anti-N-Nedd2 recognizes a major band at 53 kD in whole PC12 cell lysates (Figure 3A). The same major band was identified with the C-Nedd antibody (Figure 3B). This apparent molecular weight is in agreement with that calculated from the predicted sequence of the Nedd2 protein (51 kD). There are also 3 bands of lesser intensity seen with both antibodies at 70 kD, 60 kD and 45 kD. An identical pattern was seen with neuronally differentiated PC12 cells and a similar pattern with cultured sympathetic neurons. Specificity was assessed by absorption of the antiserum with the peptide to which it was generated and showed loss of signal by each of the above species. A minor band at 19 kD was seen on occasion at varying intensity when the N-terminal antibody was used. The major band and the additional molecular weight minor bands are down-regulated by 60-70% (n=4) after 18-22 hours treatment with V-ANedd (Figures 3A-B). In contrast, there was no down-regulation of CPP-32 on blots of the same samples probed with anti-CPP32, indicating specificity of V-ANedd treatment for Nedd2. V-SNedd, the control oligonucleotide, did not down-regulate any of the bands. None of the bands detected by Western blot appeared to be up or down regulated to a substantial degree in response to either trophic factor withdrawal or short (2-24 hrs) or long-term (10-14 days) NGF treatment. However, after withdrawal of trophic support from naive or primed PC12 cells a cleavage product of approximately 36 kD was detectable by immunoblotting with the N-terminal antiserum prior to onset of cell death.

#### Differential effects of V-ANedd on PC12 cell death

To evoke apoptotic neuronal death by trophic factor deprivation, nerve growth factor (NGF) and/or serum was withdrawn from cultures of PC12 cells (either naive, or

-25-

neuronally differentiated by NGF-pretreatment (Greene and Tischler, 1976) and neonatal rat sympathetic neurons (Rydel and Greene, 1988; Ferrari et al., 1995; Troy et al., 1996a). Oxidative stress was induced by exposing cultures to the V-linked copper/zinc superoxide dismutase (SOD1) antisense construct V-ASOD1 which down-regulates SOD1 and induces apoptosis in PC12 cells (Troy and Shelanski, 1994; Troy et al., 1996a-c). In each of these paradigms, approximately 40-60% of the cells undergo apoptosis within 24 hrs.

V-ANedd protects naive PC12 cells from death caused by serum deprivation, with maximal protection at 400 nM when added at the same time as serum withdrawal (Figures 4A-C, 7A-I). In this and all subsequent experiments, the scrambled V-SNedd construct had no effect on survival or death. Pretreatment of cultures for 4 hours with 50 nM V-ANedd shifted the dose-response curve to the left so that maximal survival was obtained with 100 nM V-ANedd. In contrast, there was no protection from SOD1 down-regulation, even at 800 nM (Figures 4A-C), and pretreatment with V-ANedd was without effect. However, V-ANedd did down-regulate Nedd2 in the presence of V-ASOD1, precluding competition by the two vector-linked constructs for cell entry. The same concentrations of V-ANedd also protected neuronally differentiated PC12 cells from apoptosis caused by NGF withdrawal (Figure 5A), but again, not from down-regulation of SOD1 (Figure 5B). Two successive additions of V-ANedd, at the time of NGF deprivation and one day later, maintained survival of greater than 75% of the cells through 4 days (Figure 5C). Although V-ANedd maintained survival, it did not mimic the actions of NGF in promoting either rapid flattening of naive PC12 cells or neurite outgrowth from neuronally-differentiated cells (Figures 7A-I).

Death of PC12 cells evoked by SOD1 down-regulation, but not by withdrawal of trophic support, is associated with enhanced

-26-

release of IL-1 $\beta$  and this is blocked by the general inhibitor of cysteine aspartase activity V-IQACRG (V-ICE<sub>inh</sub>) (Troy et al., 1996b). As illustrated in Figure 4C, V-ANedd did not affect IL-1 $\beta$  release after exposure to ASOD1. This indicates that V-ANedd does not affect processing of pro-IL-1 $\beta$  and that this is not the mechanism by which it blocks death due to trophic factor deprivation. The data in Figures 4A-C and 5A-C also show that, as expected, V-ICE<sub>inh</sub> protects cells from both trophic factor deprivation and SOD1 downregulation.

V-ANedd protects sympathetic neurons from NGF deprivation, but not from oxidative stress.

Parallel results were obtained with sympathetic neurons subjected to NGF deprivation. A single addition of V-ANedd at the time of NGF withdrawal resulted in over 60% survival after 4 days and 25% survival at 8 days; at these times, all neurons in control cultures were dead (Figures 6A-B). Although V-ANedd promoted survival, it did not maintain the neurites of NGF-deprived neurons (Figures 7A-I). Readdition of NGF to such cultures resulted in the reappearance of healthy neurites and maintenance of cell number, thereby confirming neuronal survival and function in the presence of V-ANedd.

Exposure of cultured sympathetic neurons to antisense SOD1 alone has proved insufficient to produce death, even though, as for PC12 cells, this treatment reduces SOD1 levels by 50%. In PC12 cell cultures, death due to SOD1 down-regulation requires endogenous NO (nitric oxide) synthase activity and appears due to generation of peroxynitrite (Troy et al., 1996a). Consistent with this, when V-ASOD1 and the NO generator, SNAP (S-nitrosopenicillamine) were added simultaneously to cultured sympathetic neurons, even in the presence of NGF, approximately 50% of the cells underwent apoptotic death within 24 hours. Treatment with the NO generator in the absence of SOD1 downregulation did not

-27-

produce death of either sympathetic neurons or PC12 cells (Farinelli et al., 1996). As in the study with PC12 cells (Troy et al., 1996b), the general inhibitor of cysteine aspartase activity, V-IQACRG (V-ICE<sub>inh</sub>), prevented sympathetic neuron death evoked by V-ASOD1+SNAP (Figures 6A-B). In contrast, V-ANedd was without effect in this paradigm (Figure 6B).

### Discussion

10

In the present studies, an antisense construct was designed and used to down-regulate the cysteine aspartase Nedd2 in neuronal cells and it was found that this inhibited death caused by withdrawal of trophic support, but not by oxidative stress. Multiple aspects of the studies support the specificity and utility of these reagents. The major species recognized by both our N-terminal Nedd2 antiserum and a Nedd2 C-terminal antiserum on Western blots migrated at an apparent Mr of 53 kD. This corresponds closely to the predicted Mr of the Nedd2 protein based on the sequence of the nedd2 transcript from mouse (Kumar 1995) as well as rat. Recognition of this species by anti-N-Nedd2 was abolished in the presence of excess immunizing peptide. Both antisera also provided similar patterns of cellular staining which, in the case of anti-N-Nedd2, was eliminated by preincubation with the immunizing peptide. Exposure to the V-ANedd antisense construct yielded significant down-regulation of Nedd2 protein as assessed by Western blotting and immunostaining with the two different antisera. To assess the specificity of the antisense construct, V-SNedd, a scrambled version of V-ANedd was also tested and it was observed that it did not affect either Nedd2 protein levels, staining of cells with anti-Nedd2 or cell death. Moreover, the observation that V-ANedd does not promote survival of neuronal cells after SOD1 down-regulation appears to rule out non-specific

35

-28-

anti-apoptotic actions of this construct. Finally, V-ANedd  
effectively suppressed death of serum-deprived naive PC12  
cells. In such cultures, apoptosis does not require de novo  
protein translation (Rukenstein et al, 1991), and thus this  
5 finding appears to exclude potential non-specific effects of  
the antisense construct on synthesis of proteins required for  
death.

The results of these experiments argue for the existence of at  
10 least two distinct parallel pathways to apoptotic cell death  
in the same neuron. The choice of one or the other pathway is  
a function of the initial insult to the cell. When SOD1 in  
PC12 cells is down-regulated to approximately 40% of its  
control levels, apoptosis occurs (Troy and Shelanski, 1994).  
15 This process appears to be mediated by peroxynitrite (Troy et  
al., 1996a), though the critical target of peroxynitrite in  
this model has not been identified. Cultured rat sympathetic  
neurons survive the down-regulation of SOD1 itself, but die  
rapidly when this treatment is coupled with the generation of  
20 nitric oxide. Down-regulation of SOD1 in PC12 cells is  
accompanied by an increase in the release of IL-1 $\beta$  suggesting  
the activation of an ICE-like enzyme (Troy et al., 1996b). In  
this case, death can be blocked by addition of anti-IL-1 $\beta$  or  
the IL-1 receptor antagonist (IL1-Ra) to the medium. Death of  
25 both PC12 and sympathetic neurons caused by SOD1  
downregulation can also be blocked with a variety of  
inhibitors of the ICE-family of proteases (Troy et al.,  
1996b), but interestingly, not by the down-regulation of  
Nedd2. V-ANedd does not alter the release of IL-1 $\beta$  from  
30 V-ASOD1-treated cells. These data point strongly to the  
involvement of ICE itself or an ICE-like activity in this  
model of free-radical induced cell death and appear to exclude  
an obligatory role of Nedd2.

35 In contrast to the SOD1 down-regulation paradigm, antibodies

-29-

to IL-1 $\beta$  do not rescue PC12 cells and sympathetic neurons from serum and/or trophic factor withdrawal. Moreover, the ICE antagonist peptide ZYVAD-CMK, which effectively rescues the cells from down-regulation of SOD1, has negligible effects on death provoked by loss of trophic support (Troy et al., 1996b). However, down-regulation of Nedd2 in serum deprived naive PC12 cells and in NGF-deprived primed PC12 cells and sympathetic neurons rescues them from apoptotic death pointing to a requisite role of Nedd2 in this process.

10

The extracts show a major band at 53 kD, agreeing with the predicted molecular weight of Nedd2 (Kumar et al., 1994). There are also 3 minor bands which are detected by both antibodies, two of which are higher than the calculated molecular weight for Nedd2. Although the original report on Nedd2 reported that translation of the construct resulted in a major band of 53 kD and several minor bands of 45 and 19 kD (Kumar et al., 1994) the detection of higher molecular weight bands by antibodies against both the C- and N-termini of Nedd2 and their specific down-regulation by V-ANedd strongly suggests that they are Nedd2 products. These bands are also seen after in vitro transcription translation of rat Nedd2.

15

20

Previous studies have shown that overexpression of Nedd2 can induce apoptotic death and that an antisense construct can rescue cells from apoptosis (Kumar et al., 1994; Kumar, 1995). The studies of a specifically designed compound and its inhibition of trophic withdrawal mediated cell death presented here demonstrate directly that Nedd2 protein levels are down-regulated in neuronal cells by antisense treatment and, more significantly, that Nedd2 is required for neuronal cell death resulting from trophic factor withdrawal and not required when neuronal death is induced by SOD1 down-regulation. In addition, it is shown herein that Nedd2 is processed to a 36 kD cleavage product upon withdrawal of

25

30

35

-30-

trophic support. Cleavage of Nedd2 has also been reported in another death paradigm (Srinivasan et al., 1996). ICE is proteolytically processed to an intermediate 35 kD peptide that is further cleaved to generate the active form, p20  
5 (Thornberry et al., 1992; Yamin et al., 1996). The 36 kD Nedd2 cleavage product most likely represents such an intermediate form.

The results presented herein argue against the existence of a  
10 single "final common pathway" leading to apoptotic cell death. In the two paradigms presented here, trophic factor deprivation and SOD1 down-regulation, the general scheme is similar in that each pathway requires a cysteine aspartase but shows marked selectivity in the specific enzyme required. The  
15 differential association of specific cysteine aspartases with apoptosis evoked by different means may account for the proliferation of this family in vertebrates. The utilization of distinct cysteine aspartases by the same cells to promote death from different initiating stimuli raises the possibility  
20 that this selectivity can be exploited for the treatment of specific neurodegenerative disorders.

#### Example 2 - Additional Data:

- 25 1. The human form of Nedd2, Ich1 or caspase 2, was down-regulated in Jurkat cells, a human lymphoma cell line, and found to protect the cells from anti-Fas mediated cell death in a dose-dependent manner (Figure 8).
- 30 2. Beta-amyloid peptide mediates apoptosis in PC12 cells. This apoptosis can be abrogated by down-regulation of Nedd2.

35



-31-

### Clinical Relevance of Antisense Oligonucleotides.

In the past year there have been several reports of successful utilization of antisense oligonucleotides in the treatment of disease in human clinical trials. Some reviews of this work are listed below.

1. Benner et al. J. Pharmaceol. & Toxicol. Methods 37: 229-235, 1997 review the use of antisense oligonucleotides to down-regulate genes involved in the transformation or perpetuation of hematological malignancies. Antisense oligonucleotides are being combined with conventional chemotherapy to manage the malignancies.
2. Ho PT, Parkinson, DR Seminars in Oncology 24: 187-202, 1997 reviews the use of antisense oligonucleotides as therapeutics for malignant diseases and discusses the clinical trials using antisense oligonucleotides directed against p53, bcl-2, raf kinase, protein kinase c-alpha, c-myc.
3. Oberbauer R. Wiener Klinische Wochenschrift 109: 40-46, 1997 discusses the use of antisense oligonucleotides in cardiovascular medicine, oncology and virology.

### Potential Uses

In light of the increasing use of antisense oligonucleotides as potential therapeutic agents there appears to be a real possibility that antisense oligonucleotides will play a role as specific drugs. The data which is shown in Figure 8, in the human cell line (Jurkat) show that the methods included hereinabove can be utilized for protection of many different

-32-

cells from death. Specifically, cell death may be prevented not only in neuronal cells, but also in other human cells, such as lymphoid cells and therefore likely other immune cells by administering to a subject a therapeutically effective amount of antisense oligonucleotide, such as V-ANedd or V-AICH. The sequence of V-AICH is Seq ID No. 2. The Jurkat cell line is used extensively in the study of apoptosis, particularly as a cell model system for studying cell death involving malignancies and immune-related cell death. This in vitro cell death model is known to those of skill in the art and correlates to human manifestations of diseases such as immune-related diseases and apoptosis-related diseases. Such diseases include dysfunction of the immune system, hepatitis and psoriasis. The results presented here suggest that down-regulation of caspase 2 can be protective against death of non-neuronal cells as well as death of neuronal cells. Cell death ultimately contributes to death in humans, therefore such a therapeutic method would be of great clinical interest.

Use of antisense oligonucleotide molecules in prevention or treatment of amyloidosis in a subject

V-AICH or V-ANedd or other antisense oligonucleotide molecules described herein may be administered to a subject in a therapeutically effective amount so as to prevent or treat amyloidosis in a subject. Amyloid peptide-mediated cell death has been shown in neuronal cells, such as in Alzheimer's disease (AD). Currently the etiology of neuronal death in AD is unknown but amyloid is believed to play a significant role. The protection afforded by down-regulation of caspase 2 against amyloid death indicates that it is possible to abrogate some of the neuronal cell loss which occurs in AD which would therefore, reduce or inhibit symptoms of AD in humans. This method would be of great therapeutic benefit to humans.

-33-

The antisense oligonucleotides of the present invention may be useful in preventing amyloidosis in a human subject. The present invention provides for administration of an effective  
5 therapeutic amount of an antisense oligonucleotide (such as Seq. ID NO. 1 or Seq ID No. 2) to a subject so as to prevent amyloidosis. The amyloidosis may be peripheral or systemic amyloidosis (e.g., pancreatic amyloidosis, cardiovascular amyloidosis, renal amyloidosis, liver amyloidosis, brain  
10 amyloidosis (Alzheimer's Disease or cerebrovascular amyloidosis)).

Use of antisense oligonucleotide molecules for administration  
to a subject undergoing chemotherapy

15 In addition, the antisense oligonucleotides described herein may be administered to a cancer patient in conjunction with chemotherapy in order to assist in cell death. Thus, in this scenario, the antisense compounds administered would increase  
20 cell death, thereby decreasing the necessity of generalized chemotherapy treatment and likely decreasing adverse side-effects of chemotherapy in cancer patients.

## References

- 5 Batistatou A., Greene LA (1991). Aurintricarboxylic acid rescues PC12 cells and sympathetic neurons from cell death caused by nerve growth factor deprivation: correlation with suppression of endonuclease activity. *J Cell Biol* 115:461-471.
- 10 Brown Jr RH (1995) Amyotrophic lateral sclerosis: recent insights from genetics and transgenic mice. *Cell* 80:687-692.
- Coyle JT, Puttfarcken P (1993) Oxidative stress, glutamate, and neurodegenerative disorders. *Science* 262:689-695.
- 15 Farinelli SE, Park DS, Greene LA (1996) Nitric oxide delays the death of trophic factor-deprived PC12 cells and sympathetic neurons by a cGMP-mediated mechanism. *J. Neurosci.* 16:2325-2334.
- 20 Fernandes-Alnemri T, Litwack G, Alnemri ES (1994) CPP32, a novel human apoptotic protein with homology to *Caenorhabditis elegans* cell death protein *ced-3* and the mammalian IL-1 $\beta$ -converting enzyme *J. Biol. Chem.* 269:30761-30764.
- 25 Ferrari G, Greene LA (1994) Proliferative inhibition by dominant-negative Ras rescues naive and neuronally differentiated PC12 cells from apoptotic death. *EMBO J* 13:5922-5928.
- 30 Ferrari G, Yan CYI, Greene LA (1995) N-acetylcysteine (D- and L-stereoisomers) prevents apoptotic death of neuronal cells. *J Neurosci* 15:2857-2866.
- 35 Gagliardini V, Fernandez P-A, Lee RKK, Drexler HCA, Rotello RJ, Fishman MC, Yuan J. (1994) Prevention of vertebrate

-35-

neuronal death by the *crmA* gene. *Science* 263:826-828.

Greene LA (1978) Nerve growth factor prevents the death and stimulates the neuronal differentiation of clonal PC12 pheochromocytoma cells in serum-free medium. *J Cell Biol* 78:747-755.

Greene LA, Tischler AS (1976). Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. *Proc. Natl. Acad. Sci. U.S.A.* 73, 2424-2428.

Hengartner MO, Ellis RE, Horvitz HR (1992) *Caenorhabditis elegans* gene *ced-9* protects cells from programmed cell death. *Nature*. 356: 494-9

Kumar S (1995) Inhibition of apoptosis by the expression of antisense *Nedd2*. *FEBS Letters* 368:69-72.

Kumar S, Knioshita M, Noda M, Copeland NG, Jenkins NA (1994) Induction of apoptosis by the mouse *Nedd2* gene, which encodes a protein similar to the product of the *Caenorhabditis elegans* cell death gene *ced-3* and the mammalian IL-1 $\beta$ -converting enzyme. *Genes & Develop* 8:1613-1626.

Lindenboim L, Haviv R, Stein R (1995) Inhibition of drug-induced apoptosis by survival factors in PC12 cells. *J Neurochem* 64:1054-1063.

Mesner PW, Winters TR, Green SH (1992) Nerve growth factor withdrawal-induced cell death in neuronal PC12 cells resembles that in sympathetic neurons. *J Cell Biol* 119:1669-1680.

Miura M, Zhu H, Rotello R, Hartweig EA, Yuan J (1994) Induction of apoptosis in fibroblasts by IL-1 $\beta$ -converting

-36-

enzyme, a mammalian homolog of the *c. elegans* cell death gene *ced-3*. *Cell* 75:653-660.

5 Pittman RN, Wang S, DiBenedetto AJ, Mills JC (1993) A system for characterizing cellular and molecular events in programmed neuronal cell death. *J Neurosci* 13:3669-3680.

10 Rukenstein A, Rydel RE, Greene, LA (1991) Multiple agents rescue PC12 cells from serum-free cell death by translation- and transcription-independent mechanisms. *J Neurosci* 11:2552-2563.

15 Rydel RE, Greene LA (1988) cAMP analogs promote survival and neurite outgrowth in cultures of rat sympathetic and sensory neurons independently of nerve growth factor. *Proc Natl Acad Sci USA* 85:1257-1261.

20 Schapira AHV (1995) Oxidative stress in parkinson's disease. *Neuropath and Appl Neurobiol* 21:3-9.

25 Srinivasan A, Foster LM, Testa M-P, Ord T, Keane RW, Bredesen DE, Kayalar, C (1996) Bcl-2 expression in neural cells blocks activation of ICE.CED-3 family proteases during apoptosis. *J Neurosci* 16: 5654-5660.

30 Theodore L, Derossi D, Chassaing G, Llibat B, Kubes M, Jordan P, Chneilweiss H, Godement P, Prochiantz A (1995) Intraneuronal delivery of protein kinase C pseudosubstrate leads to growth cone collapse. *J Neurosci* 15:7158-7167.

35 Thornberry NA, Bull HG, Calaycay JR, Chapman KT, Howard AD, Kostura MJ, Miller DK, Molineaux SM, Weidner JR, Aunins J, et al. (1992) A novel heterodimeric cysteine protease is required for interleukin-1 beta processing in monocytes. *Nature*. 356:768-74.

-37-

- Troy CM, Brown K, Greene LA, and Shelanski ML (1990). Ontogeny of the neuronal intermediate filament protein, peripherin, in the mouse embryo. *Neurosci.* 36: 217-237.
- 5 Troy CM, Derossi D, Prochiantz A, Greene LA, Shelanski ML (1996a) Down-regulation of SOD1 leads to cell death by the NO-peroxynitrite pathway. *J Neurosci* 16:253-261.
- Troy CM, Greene LA, Shelanski ML (1992). Neurite outgrowth  
10 in peripherin-depleted PC12 cells. *J Cell Biol* 117:1085-1092.
- Troy CM, Shelanski ML (1994) Down-regulation of copper/zinc superoxide dismutase (SOD1) causes neuronal cell death. *Proc. Natl. Acad. Sci. USA* 91:6384-6387.
- 15 Troy CM, Stefanis L, Prochiantz A, Greene LA, Shelanski ML (1996b) The contrasting roles of ICE-family proteases and interleukin-1 $\beta$  in apoptosis induced by trophic factor withdrawal and by SOD1 downregulation. *Proc Natl Acad Sci USA*  
20 93: 5635-5640.
- Troy CM, Stefanis L, Greene L A, Shelanski ML (1996c) Mechanisms of neuronal degeneration: a final common pathway? in *Neuronal Regeneration, Reorganization and Repair* F. Seil, ed. (New York, NY: Raven Press), pp. 103-112.  
25
- Wang L, Miura M, Bergeron L, Zhu H, Yuan J (1994) Ich-1, an Ice/ced-3-related gene, encodes both positive and negative regulators of programmed cell death. *Cell* 78: 739-750.
- 30 Williams LR (1995) Oxidative stress, age-related neurodegeneration, and the potential for neurotrophic. *Cerebrovasc. Brain Metab. Rev.* 7:55-73.
- 35 Yamin T-T, Ayala JM, Miller DK (1996) Activation of the

-38-

native 45-kDa precursor form of interleukin-1-converting enzyme. J Biol Chem 271: 13273-13282.

5 Yuan J, Shahan S, Ledoux S, Ellis HM, Horvitz HR (1993) The c. elegans cell death gene ced-3 encodes a protein similar to mammalian interleukin-1 $\beta$ -converting enzyme. Cell 74: 641-652.



-39-

What is claimed is:

1. An oligonucleotide having the sequence  
5'GCTCGGCGCCGCCATTTCCAG3' (SEQ ID No. 1).
2. An oligonucleotide having the sequence  
5'GTCAGCGGCCATCAGCTT3' (Seq ID No. 2).
3. A compound comprising the oligonucleotide of claim 1 or  
2, and an agent capable of specifically directing the  
compound to a cell.
4. The compound of claim 3, wherein the agent comprises a  
portion of an Antennepedia polypeptide.
5. The compound of claim 3, wherein the agent comprises at  
least a portion of a polypeptide comprising the  
sequence NPyS-Arg-Gln-Ile-Lys-Ile-Trp-Phe-Gln-Asn-Arg-  
Arg-Met-Lys-Trp-Lys-Lys- (Seq. I.D. No. 3).
6. The compound of claim 3, wherein the agent comprises  
an antibody, an adjuvant or a cell-specific ligand.
7. The compound of claim 3, wherein the agent comprises  
Penetratin<sup>®</sup>.
8. The compound of claim 3, wherein the cell is a neuronal  
cell.
9. A pharmaceutical composition comprising the  
oligonucleotide of claim 2 and a pharmaceutically  
acceptable carrier.
10. The pharmaceutical composition of claim 9, wherein the  
carrier comprises a diluent, an appropriate adjuvant,

-40-

a herpes virus, a liposome, a microencapsule, a neuronal cell receptor ligand, a neuronal-specific virus, a polymer encapsulated cell or a retroviral vector.

5

11. The pharmaceutical composition of claim 9, wherein the pharmaceutically acceptable carrier is an aerosol, intravenous, oral or topical carrier.

10

12. A method of inhibiting trophic factor withdrawal mediated death of a cell which comprises contacting the cell with an amount of the oligonucleotide of claim 1 effective to inhibit death of the cell.

15

13. The method of claim 12, wherein the cell is in a subject.

14. The method of claim 12, wherein the subject is a human.

20

15. The method of claim 12, wherein the cell is a neuronal cell.

16. The method of claim 12, wherein the agent comprises a polypeptide comprising at least a portion of an Antennapedia polypeptide.

25

17. The method of claim 12, wherein the agent comprises a polypeptide having the sequence NPyS-Arg-Gln-Ile-Lys-Ile-Trp-Phe-Gln-Asn-Arg-Arg-Met-Lys-Trp-Lys-Lys- (Seq. I.D. No. 3).

30

18. The method of claim 12, wherein the agent comprises an antibody, an adjuvant or a cell-specific ligand.

35

19. A method for treating a neurodegenerative disorder in

-41-

a subject which comprises administering to the subject the compound of claim 3, the compound being present in an amount effective to inhibit neuronal cell death and thus treat the neurodegenerative disorder in the subject.

5

20. The method of claim 19, wherein the neurodegenerative disorder is associated with aging, amyotrophic lateral sclerosis, Alzheimer's disease, dentatorubral and pallidolysian atrophy, Huntington's disease, Machado-Joseph disease, multiple sclerosis, muscular dystrophy, Parkinson's disease, senility, spinocerebellar ataxia type I, spinobulbar muscular atrophy, stroke, trauma.

10

21. The method of claim 20, wherein the administration comprises aerosol delivery; intralesional, intraperitoneal, intramuscular or intravenous injection; infusion; liposome-mediated delivery; anal, nasal, oral, ocular, otic or topical delivery of the pharmaceutical composition.

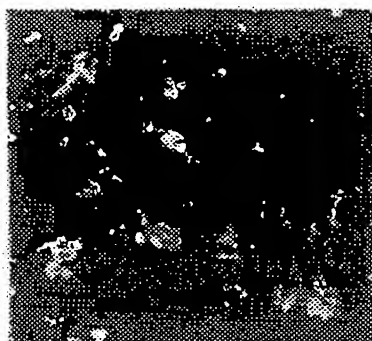
15

20

1/15

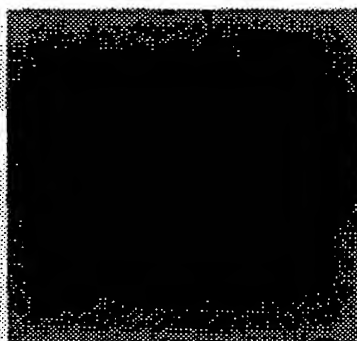
**FIG. 1A**

Control



**FIG. 1B**

V-ANedd treated



Naive  
PC12 cells

2/15

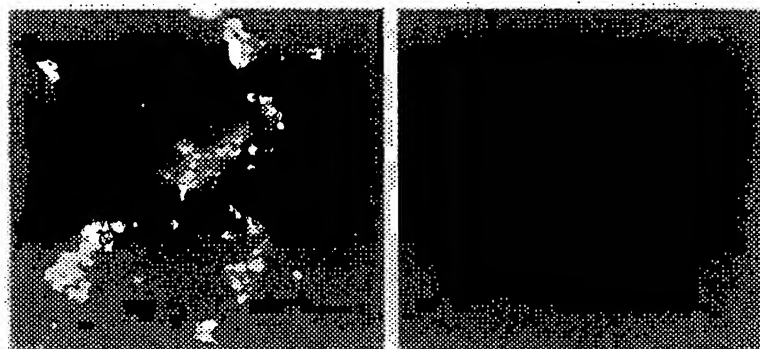
FIG. 1C

FIG. 1D

Control

V-ANedd treated

Neuronal  
PC12 cells

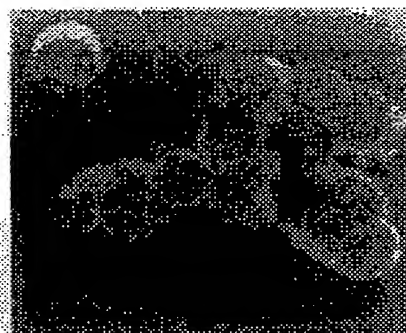


3/15

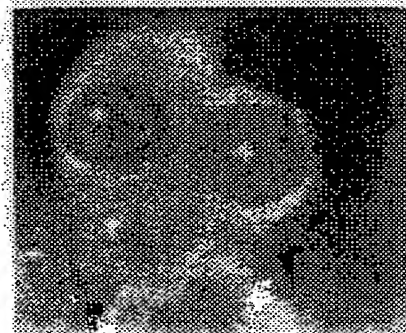
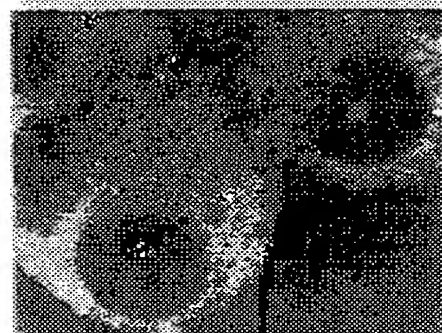
**FIG. 2A**  
**+NGF**

**FIG. 2B**  
**-NGF**

**Naive**  
**PC12 cells**



**Neuronal**  
**PC12 cells**



**FIG. 2C**

**FIG. 2D**

4/15

FIG. 3A

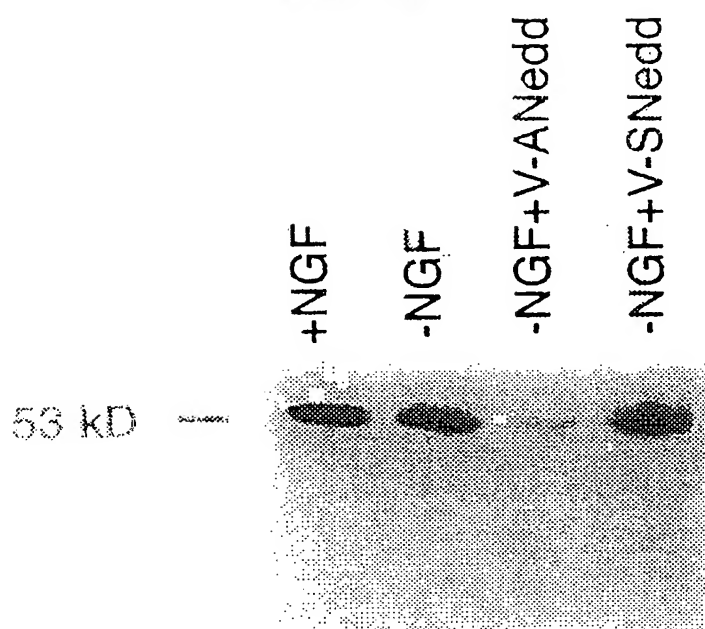
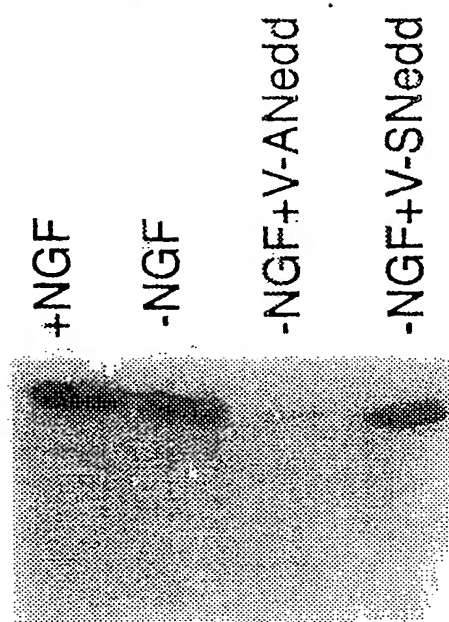
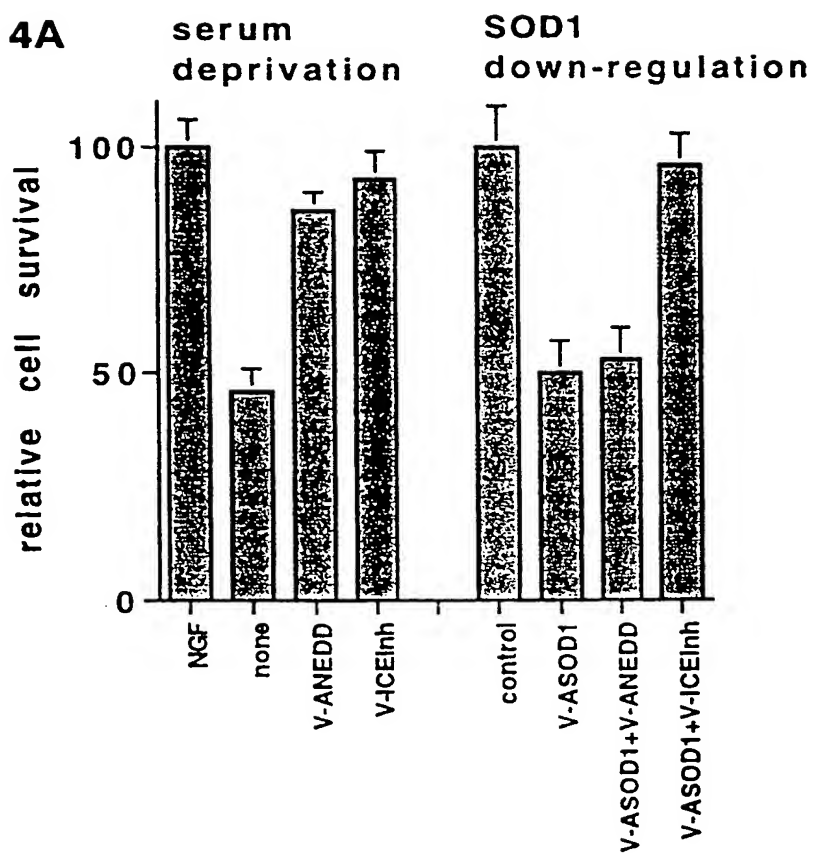


FIG. 3B



5/15

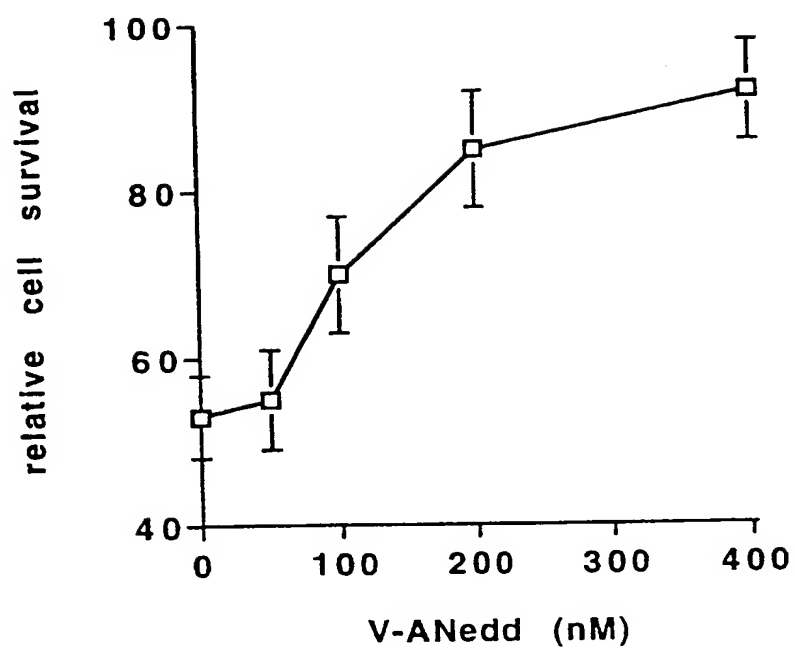
FIG. 4A





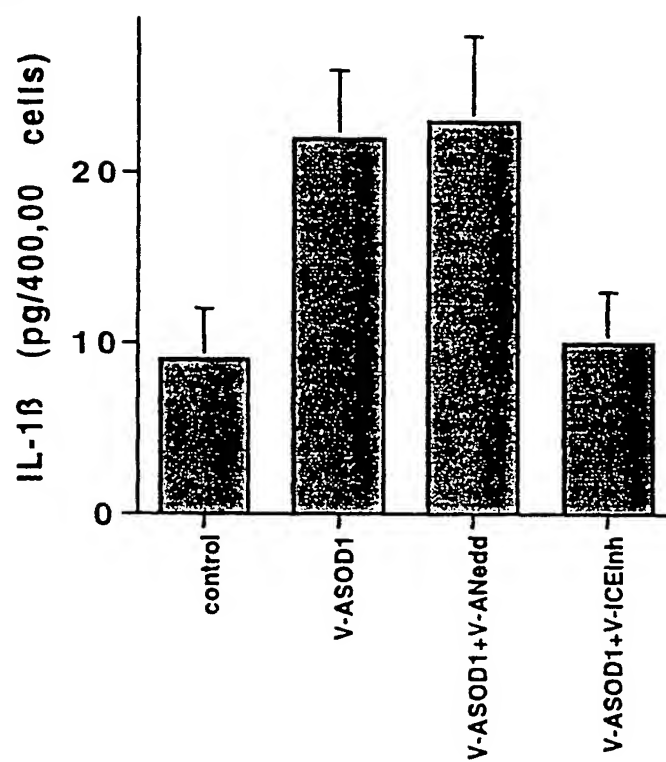
6/15

FIG. 4B



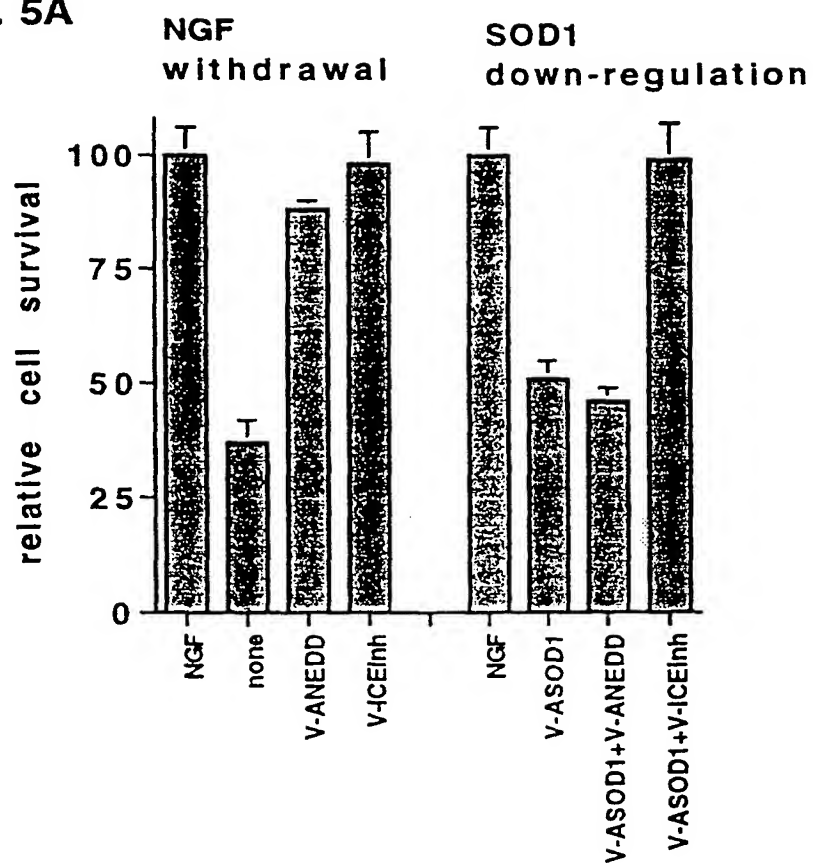
7/15

FIG. 4C



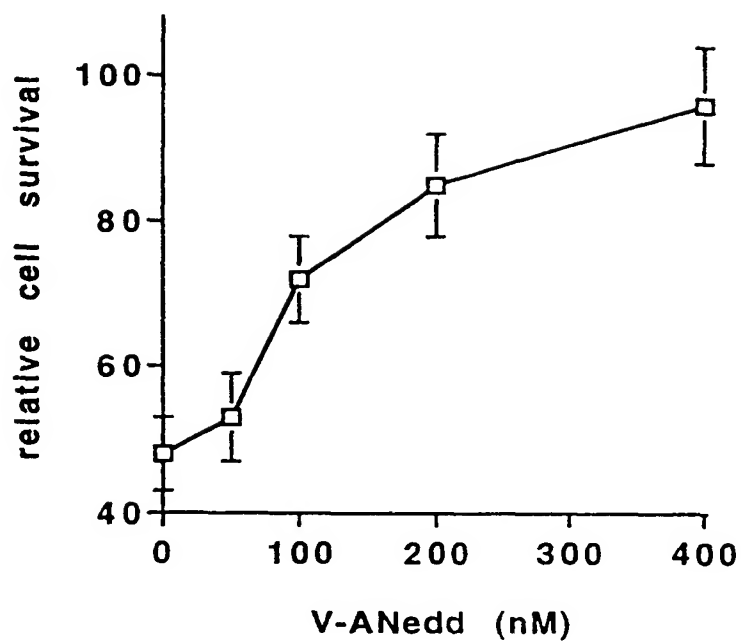
8/15

FIG. 5A



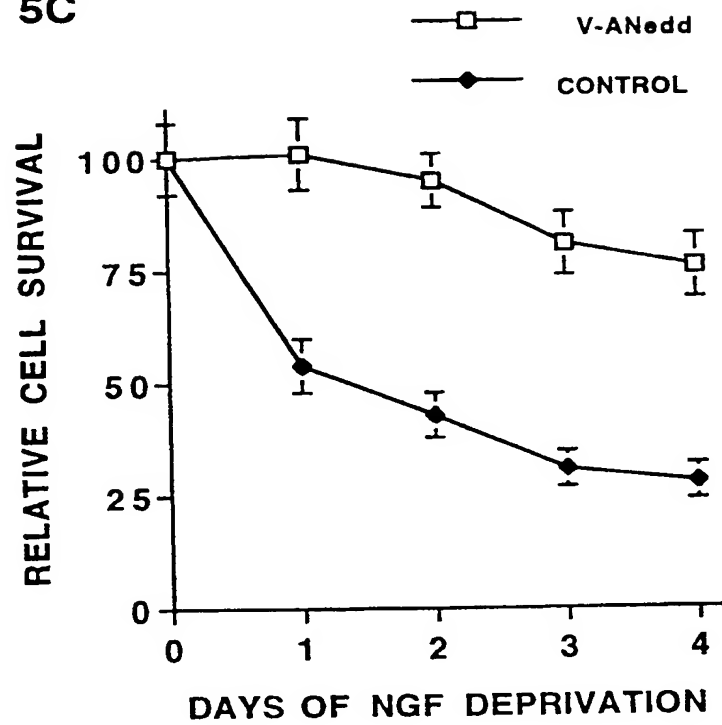
9/15

FIG. 5B



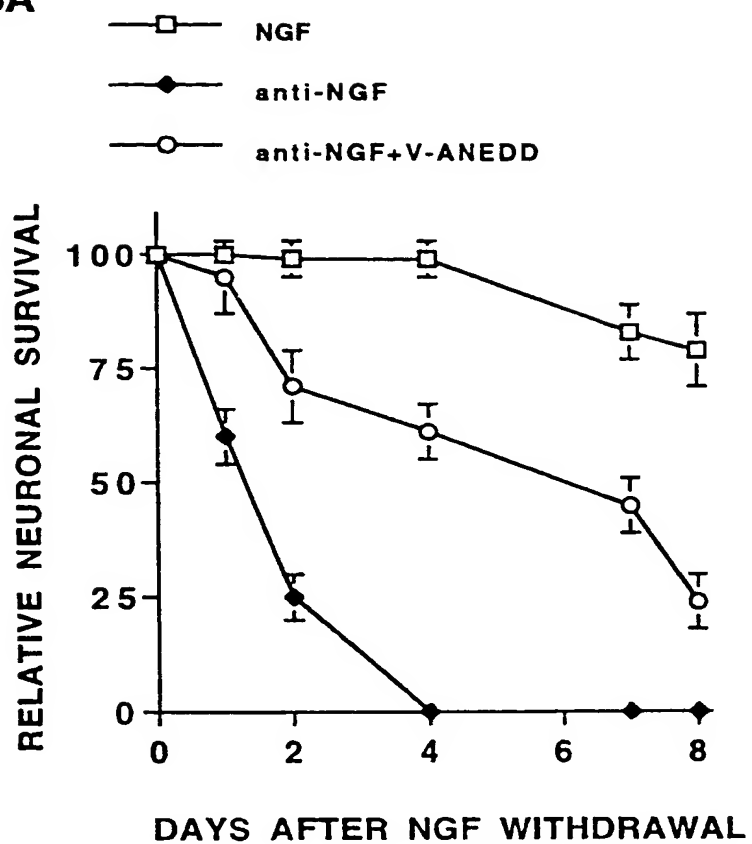
10/15

FIG. 5C



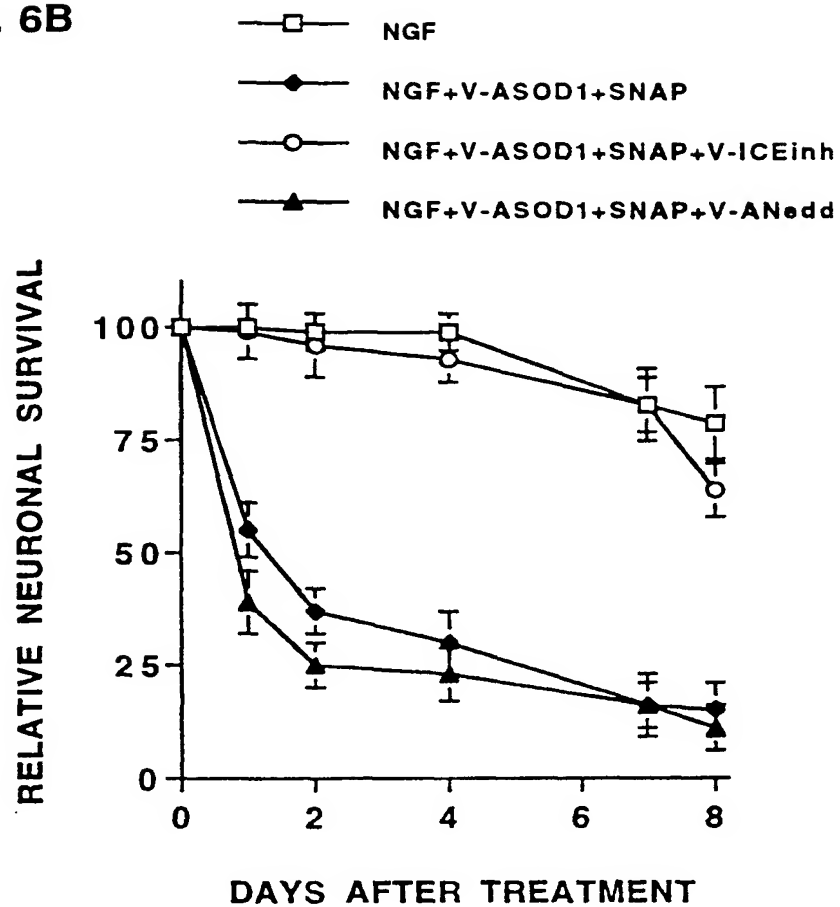
11/15

FIG. 6A

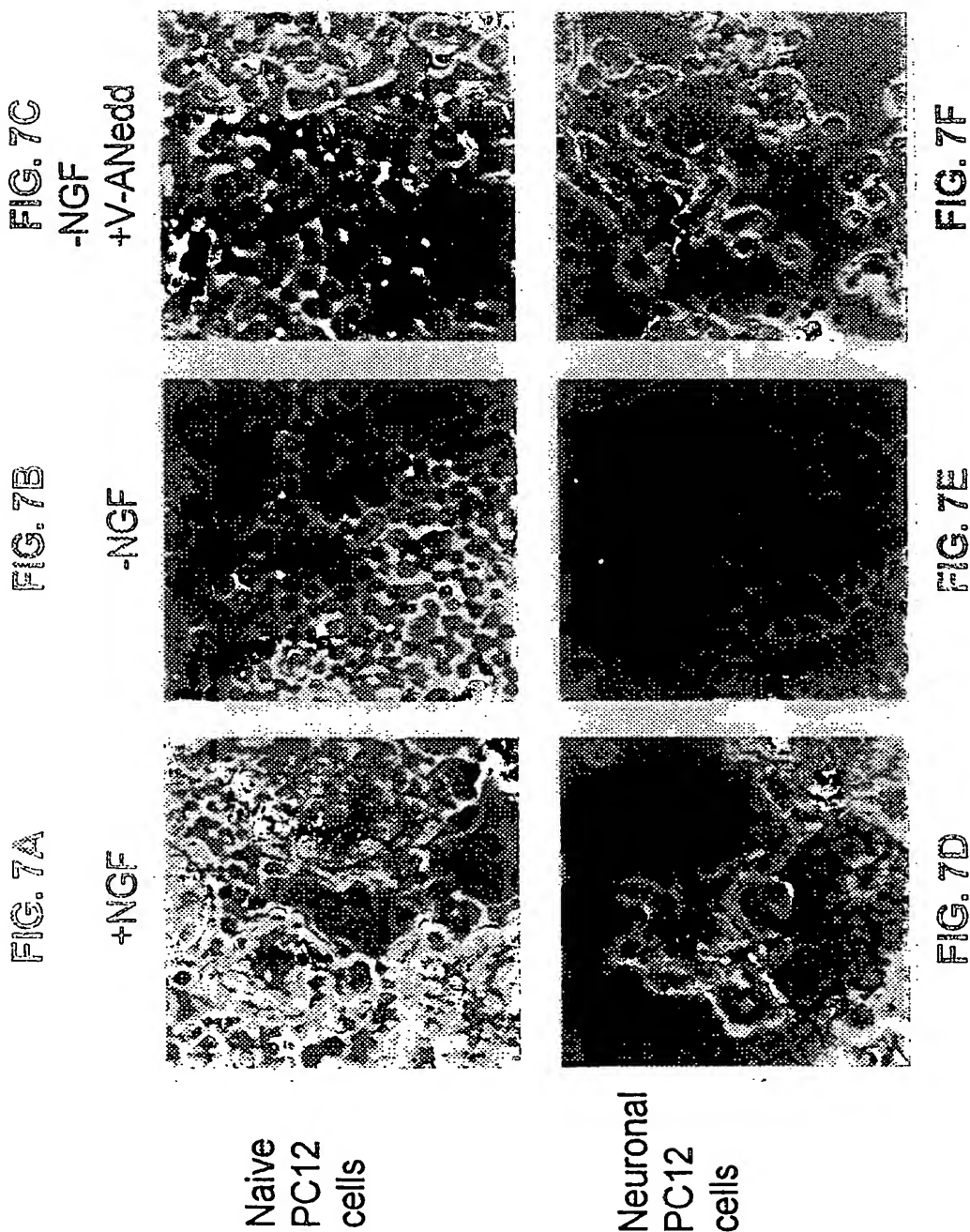


12/15

FIG. 6B



13/15





14/15

FIG. 7I  
-NGF  
+V-ANedd

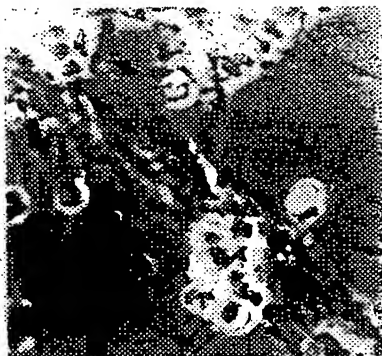
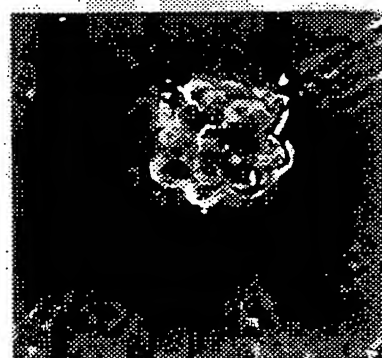


FIG. 7H  
-NGF



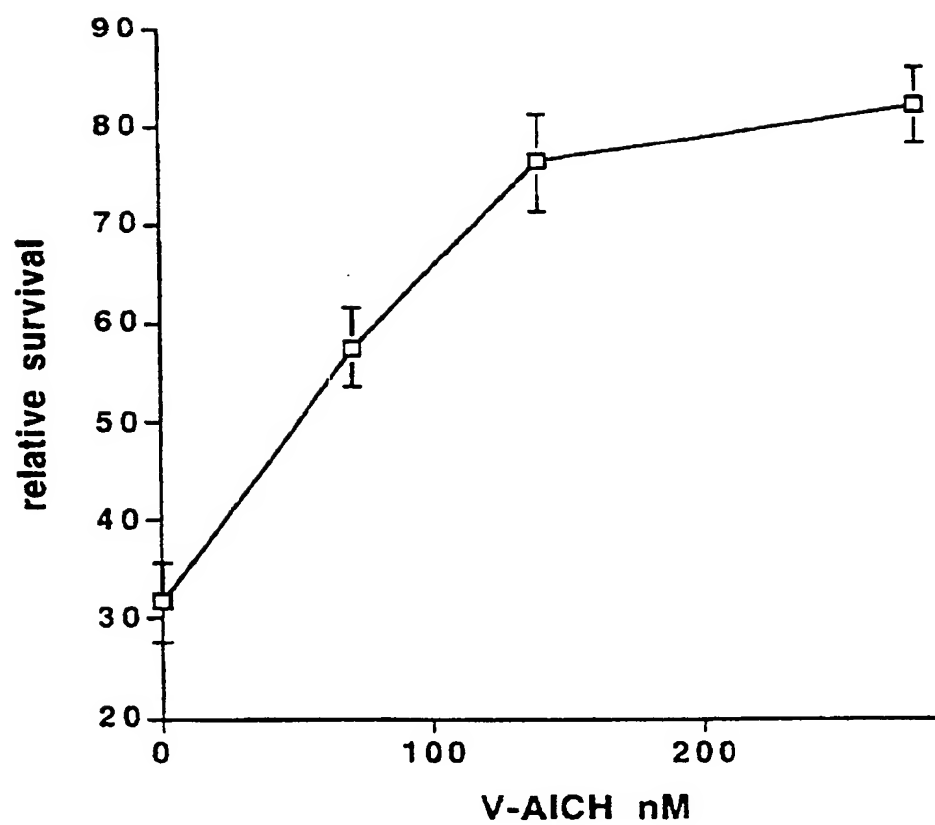
FIG. 7G  
+NGF



SCG  
neurons

15/15

FIG. 8



## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US98/04128

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : A01N 43/04; C12Q 1/68; C12P 21/06; C12N 15/00

US CL : 514/44; 435/6, 69.1, 172.3

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/44; 435/6, 69.1, 172.3

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched  
NONEElectronic data base consulted during the international search (name of data base and, where practicable, search terms used)  
Please See Extra Sheet.

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 95/00160 A1 (THE GENERAL HOSPITAL CORPORATION) 05 January 1995, see entire document.	1-21
Y	WO 96/00297 A1 (HUMAN GENOME SCIENCES, INC.) 04 January 1996, see entire document.	1-21
Y	DORN et al. Homeodomain Proteins in Development and Therapy. Pharmacology & Therapeutics. 1994, Vol. 61, No. 1/2, pages 155- 183, see especially pages 163, 166, 172 and 175.	4 and 16
Y	PROCHIANTZ, A. Getting Hydrophilic Compounds into Cells: Lessons from Homeopeptides. Current Opinion in Neurobiology. October 1996, Vol. 6, No. 5, pages 629-634, see entire document.	4 and 16

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A* document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*E* earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*I* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*Z* document member of the same patent family
*O* document referring to an oral disclosure, use, exhibition or other means	
*P* document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

06 APRIL 1998

Date of mailing of the international search report

01 JUL 1998

Name and mailing address of the ISA/US  
Commissioner of Patents and Trademarks  
Box PCT  
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

IREM YUCEL

Telephone No. (703) 308-0196

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US98/04128

## B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

APS, STN, DIALOG, Caplus, Medline, WPID

search terms: inventors' names, apoptosis, cell death, protein?, ICE, interleukin converting, Nedd2, ced, antennapedia, transport, inhibit? express?